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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT** Expression of the seven transmembrane-spanning receptor (7TMR), GPR30, in primary human breast tumors is positively associated with several tumor progression variables including extra mammary metastases (Filardo et al, 2006). Altered expression of 7TMRs is linked with a spectrum of disease phenotypes, including cancer, raising the possibility that GPR30 may function as an oncogene. To test this hypothesis, two lines of transgenic mice (T6-1A and T6-2E) were engineered with stably integrated hemagglutinin-tagged (HA-GPR30) transgenes under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter. Endogenous GPR30 protein was readily detected in mammary glands harvested from control and transgenic mice using peptide antibodies. However, breast tissue from nulliparous progeny mice expressed no detectable GPR30 transgene protein or mRNA. MMTV-HA-GPR30 mice exhibited normal reproductive behavior, lactational competence and no overt signs of cancer or premaligancy. Increased transgene expression or abnormal mammary gland growth was also not evidenced in multiparous mice. A second no cost extension was requested to construct mice with an HA-GPR30 transgene regulated by the whey acidic protein(WAP) promoter.

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membrane receptor, estrogen, transgenic mice

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## Introduction.

Breast tumor growth and survival is strongly influenced by estrogen and decisions regarding appropriate adjuvant therapy for patients with breast cancer are largely determined by the measurement of known estrogen receptors (ERs) in primary tumor biopsy specimens. However, it has long been suspected that receptors other than the known estrogen receptors may promote estrogen action. Recent findings by our lab (1-4), and others (5-8), has shown that the seven transmembrane receptor (7TMR), GPR30, promotes specific estrogen binding and biochemical signaling and in addition is linked to tumor progression in man. To further address the role of GPR30 in experimental breast tumor biology, we have proposed to generate transgenic mice capable of overexpressing wild type or active GPR30 using mammary gland specific promoters.

## Body.

### Work conducted during no cost extension.

The one-year term of this Concept award expired on October 31, 2007. The overall goal of the concept award was to generate transgenic mice that overexpressed wild-type or active GPR30 for the purpose of assessing the biological role of this newly appreciated membrane estrogen receptor in breast cancer. We successfully generated two founder mice (T6-1A and T6-2E) that contained a stably integrated wild-type hemagluttinin (HA)- tagged GPR30 transgene during that time frame and were unable to identify an active GPR30 allele (see Nov 1, 2006- Oct 31, 2007 progress report). Accordingly, we requested and were granted, a no-cost extension to further evaluate the expression of the transgene in these mice and to determine if there was an association with the development of mammary adenocarcinoma.

The work performed during the no cost extension was limited to Task 1 of the original *Statement of Work*. No efforts were made to pursue the development of an active GPR30 allele as remaining monies did not allow. No salary support was drawn during the no-cost extension. Monies budgeted for the generation of GPR30 CAM mice were used, in part, to conduct breeding experiments that were designed to assess MMTV-HA-GPR30 transgene expression and potential malignancy in the mammary glands of nulliparous, parous and multiparous mice. The results of these experiments are listed below.

***Salaries were covered entirely by Departmental Funds from Medicine at Rhode Island Hospital.***

### Task 1. To evaluate the impact of hyperexpressed wild-type GPR30 on mammary duct branching and predisposition for the development of invasive breast cancer.

### Expression of MMTV-HA-GPR30 in T6-1A and T6-2E transgenic mouse strains.

Two lines of mice (T6-1A and T6-2E) harboring stably integrated MMTV-HA-GPR30 transgenes were evaluated for transgene expression. The mouse mammary tumor virus (MMTV) promoter has been used extensively to conditionally express transgenes in the mammary gland (*reviewed* in 9,10). Some variation in

expression has been reported based upon the content of the transgene (*cis* effect) and parity (*trans* effect). To evaluate the expression of the HA-GPR30 in each of these transgenic lines and the potential influence of pregnancy on transgene expression, we generated transgene positive female progeny and then evaluated the expression of HA-GPR30 in nonporous, parous and multiparous mice.

Despite the fact that the transgene was stably inherited, the MMTV-HA-GPR30 transgene protein was not detected in either lineage and was not influenced by the pregnancy status of the mice (**figure 1**). The HA-GPR30 gene product was readily detected when expressed under a CMV promoter in HEK-293 cells suggesting that the inability to express the MMTV HA-GPR30 transgene in mice was related to the promoter (**figure 2**). No indications of preneoplasia or malignancy were observed in any of the transgene positive mice. However, due to the fact that we did not measure transgene expression, we are unable to conclude whether GPR30 is involved in spontaneous mammary adenocarcinoma.

## Key Research Elements.

None.

## Reportable Outcomes.

None.

## Conclusions.

Despite the fact that the transgene was stably inherited in two lines of MMTV-HA-GPR30 mice, the MMTV-HA-GPR30 transgene protein was not detected in either lineage and was not influenced by the pregnancy status of the mice. The HA-GPR30 gene product was readily detected when expressed under a CMV promoter in HEK-293 cells suggesting that the inability to express the MMTV HA-GPR30 transgene in mice was related to the promoter. We have previously shown in work supported internally here at Rhode Island Hospital that WAP-regulated carboxyl truncated HA-GPR30 protein is efficiently expressed in the mammary glands of mice (**figure 3**). We originally proposed to employ a WAP expression vector in our original *Statement of Work* but decided against this based upon the fact that while transgene expression was extremely abundant (as assessed by immunohistochemistry with HA antibodies), the percentage of mammary ducts positive for the transgene varied among litter mates. However, this difference may be overlooked if any positive results are generated demonstrating an association between GPR30 and the development of spontaneous mammary adenocarcinoma. This work is worth pursing since we (4), and others (8), have shown a strong association between GPR30 expression and malignancy in breast and uterine neoplasia in man.

Since, we have clearly shown that the MMTV-HA-GPR30 transgene was not expressed in these mice and therefore cannot make any conclusions about the role of GPR30 in spontaneous murine mammary adenocarcinoma, a second no cost extension was requested to engineer mice that express HA-GPR30 under the influence of a WAP promoter. No additional monies are requested for this work, and all salaries will be supported by internal Funds. This work is significant in that these animals may represent potential interesting models for studying the role of this previously unappreciated estrogen receptor in breast cancer biology.

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## Appendices.

None.

**Supporting data (figures 1-3).**

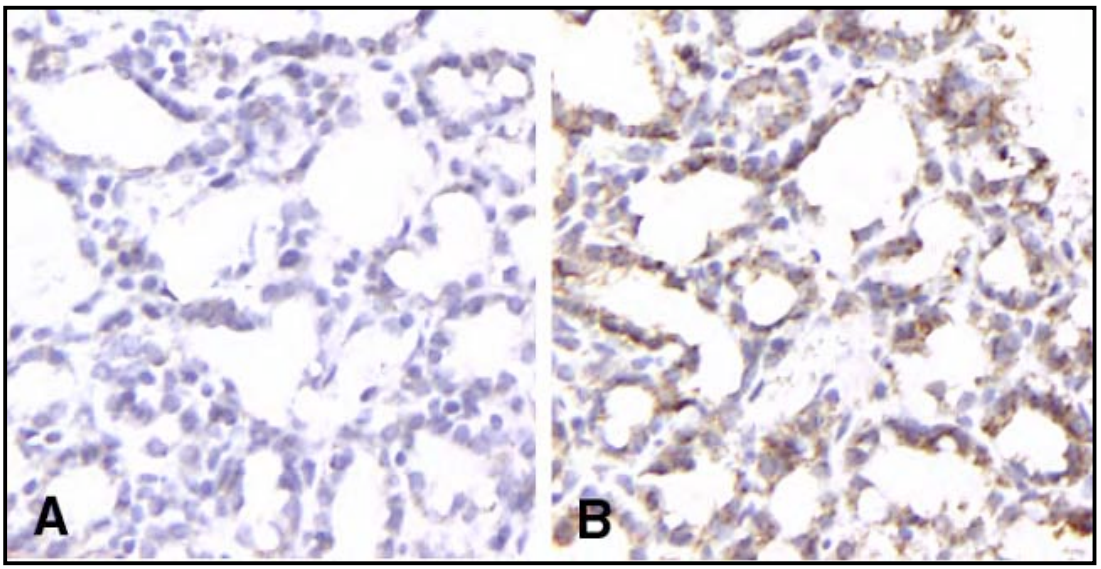


**Figure 1. Failure to express HA-GPR30 protein in mammary glands from MMTV-HA-GPR30 mice.** Mammary glands derived from HA-GPR30 transgene positive mouse, T12-1G, that was immunostained with (A) HA-antibodies or (B) C-ter GPR30 peptide antibodies. Images are shown at 100X.

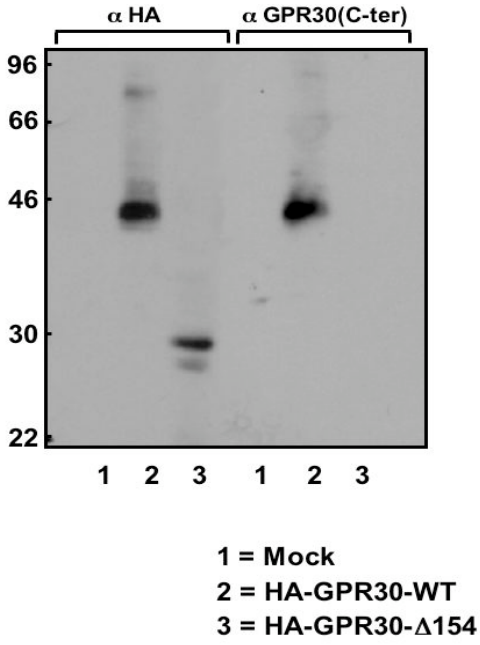


**Figure 2. Expression of HA-GPR30 protein.** Total protein (25 μg) from HEK-293 cells transfected with: vector, HA-GPR30, or C-terminally truncated HA-GPR30 immunoblotted with anti-hemagluttinin (HA) or GPR30 C-TER peptide antibodies. Molecular mass standards are indicated at left (kDa).

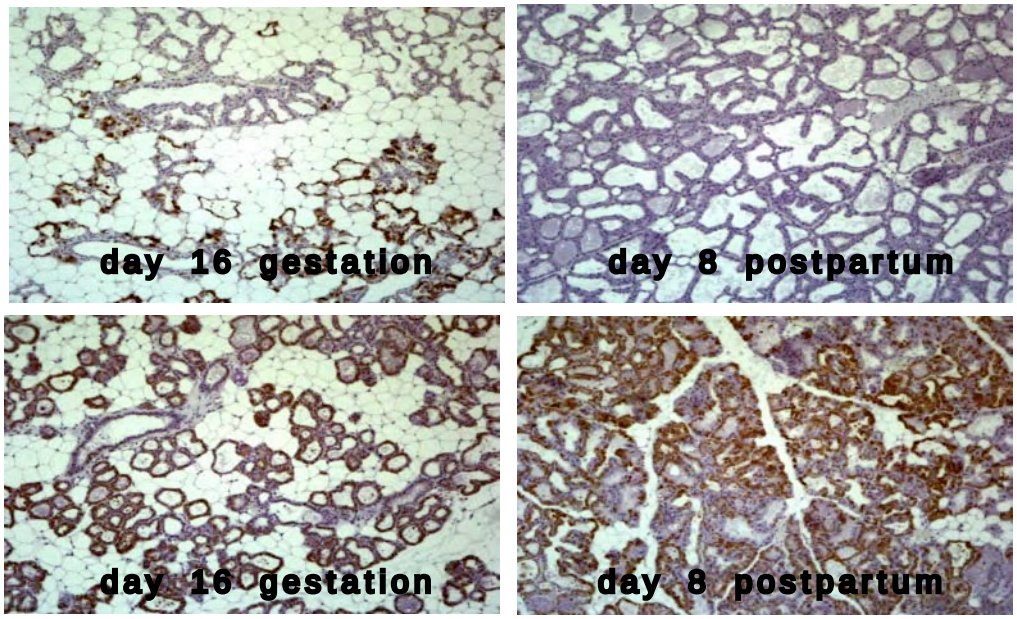


**Figure 3. Expression of HA-GPR30Δ154 carboxyl-terminated protein in mammary glands from WAP-HA-GPR30Δ154 mice.** Mammary glands from 4 individual transgene positive WAP-HA-GPR30Δ154 mice from the same founder on day 16 of gestation and from day 8 postpartum immunostained with HA antibodies. While transgene expression is strong, notice the variation in the percentage of mammary duct lobules that positively express the transgene product.